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Proton Transfer in the K-Channel Analog of B-Type Cytochrome *c* Oxidase from *Thermus thermophilus*

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ABSTRACT A key enzyme in aerobic metabolism is cytochrome *c* oxidase (C*c*O), which catalyzes the reduction of molecular oxygen to water in the mitochondrial and bacterial membranes. Substrate electrons and protons are taken up from different sides of the membrane and protons are pumped across the membrane, thereby generating an electrochemical gradient. The well-studied A-type C*c*O uses two different entry channels for protons: the D-channel for all pumped and two consumed protons, and the K-channel for the other two consumed protons. In contrast, the B-type C*c*O uses only a single proton input channel for all consumed and pumped protons. It has the same location as the A-type K-channel (and thus is named the K-channel analog) without sharing any significant sequence homology. In this study, we performed molecular-dynamics simulations and electrostatic calculations to characterize the K-channel analog in terms of its energetic requirements and functionalities. The function of Glu-15B as a proton sink at the channel entrance is demonstrated by its rotational movement out of the channel when it is deprotonated and by its high pK_A value when it points inside the channel. Tyr-244 in the middle of the channel is identified as the valve that ensures unidirectional proton transfer, as it moves inside the hydrogen-bond gap of the K-channel analog only while being deprotonated. The electrostatic energy landscape was calculated for all proton-transfer steps in the K-channel analog, which functions via proton-hole transfer. Overall, the K-channel analog has a very stable geometry without large energy barriers.

INTRODUCTION

A key element of aerobic metabolism is the enzyme cytochrome *c* oxidase (C*c*O), which catalyzes the reduction of molecular oxygen to water. Four electrons and four protons are transferred onto a single oxygen molecule in a stepwise reaction. The integral membrane protein C*c*O utilizes the resultant energy to pump protons across the membrane, thereby establishing an electrochemical gradient.

The C*c*O family may be divided into A, B, and C types. The well-studied A-type C*c*O is most abundant and constitutively expressed in mitochondria and several bacteria (1,2), whereas B- and C-type C*c*O are expressed in some specialized bacteria under low-oxygen conditions (3). Whereas the C-type C*c*O is more distant to the others (4), the A and B types share higher sequence similarity and have almost identical compositions of cofactors used for electron transport and enzymatic reaction (5,6). At the positively charged P side of the membrane, C*c*O takes up electrons from soluble cytochrome *c*. They are transported via the bimetallic copper A center (Cu_A) to heme a (A type) or heme b (B type), and finally to the binuclear center (BNC), where the chemical reaction takes place. The BNC is composed of heme a_3 and the copper B center (Cu_B), and has a nearly identical three-dimensional structure in both A- and B-type C*c*O (5,6). The proton-pumping efficiency differs between the A- and B-type C*c*O (1 H^+_{pump}/e^- and 0.5 H^+_{pump}/e^-, respectively) (7), although it was speculated that the B-type pump efficiency may be underestimated due to experimental limitations (8). The intermediate states of the catalytic cycle in A-type C*c*O, which have been investigated intensively (2,9), may differ from those in the B type (10). Also, the proton input pathways of A- and B-type C*c*O differ from each other. All protons are taken up from the negatively charged N side of the membrane and can be differentiated into chemical protons that are consumed in the reaction and pumped protons, which are pumped across the membrane. In the A-type C*c*O, the protons are conducted via two different pathways (1,2): 1), the D-channel leading from aspartate (Asp-132, *R. sphaeroides* numbering) at the entrance to glutamate (Glu-286) situated between the hemes; and 2), the K-channel containing lysine (Lys-362) and terminating at tyrosine (Tyr-288), which is covalently bound to His-284 ligating Cu_B. For the A-type C*c*O, it is well established that all pumped protons are taken up via the D-channel, and that two chemical protons enter via the D-channel and two enter via the K-channel. In contrast, the B-type C*c*O was shown to transport all chemical and pumped protons via the same input channel (11), which is located at a similar position as the K-channel of the A-type C*c*O (6) without sharing significant sequence homology. This K-channel analog in B-type C*c*O (Fig. 1) involves a hydrogen bond (H-bond) chain of threonines, tyrosines, and serine, and a water molecule with only one

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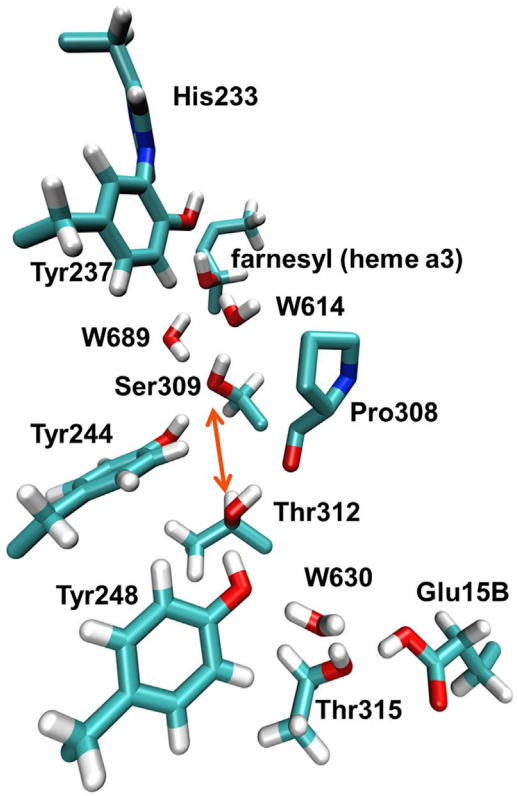

FIGURE 1 Proton wire in the K-channel analog in C*c*O of *T. thermophilus*. The channel runs from Glu-15B to Tyr-237, with one gap of 4.9 Å in the H-bond chain between Thr-312 and Ser-309 (indicated by an *orange arrow*). For proton transport, a proton hole is presumably transported from Tyr-237 to Glu-15B via farnesyl, W614, Ser-309, Tyr-244, Thr-312, Tyr-248, W630, and Thr-315. Coordinates were taken from crystal structure PDB 3S8F (6) and hydrogen atoms were added with CHARMM (19). In addition to the channel residues, Pro-308 is depicted (without hydrogens) because its backbone CO group accepts an H-bond from Thr-312, and His-233 is depicted because it is covalently bound to Tyr-237. For clarity, the farnesyl chain of heme a_3 is shown only partially. To see this figure in color, go online.

4.9 Å gap in between Thr-312 and Ser-309 (Fig. 1). Only glutamate and tyrosine at the entrance and terminus, respectively, are conserved among the K-channels of A- and B-type C*c*O.

For the A-type C*c*O, the properties of the D-channel for collecting and gating protons have been discussed extensively (12–14), and recently we also found a gating element in the K-channel (15). In the B-type C*c*O, these functionalities must be accomplished by a single proton input channel that ensures rapid unidirectional proton flow and translocation of pumped protons.

In this study, we use molecular-dynamics (MD) simulations and electrostatic energy computations to explore the energy landscape of proton transfer via the K-channel analog in the B-type C*c*O. We highlight the key functionalities of specific molecular geometries and offer an explanation for how the channel may ensure proton transport that is rapid as well as unidirectional.

MATERIALS AND METHODS

Preparing and performing MD simulations of C*c*O

The coordinates of subunits I, II, and IIa of C*c*O from *Thermus thermophilus* were taken from the Protein Data Bank (16) (PDB ID: 3S8F (5)), embedded in a lipid bilayer of phosphatidylcholines modeled with the plug-in of VMD (17), and solvated in a TIP3P (18) water box applying periodic boundary conditions (dimensions: 100 Å × 100 Å × 107.8 Å). We employed the CHARMM22 force field (19), CHARMM36 extension for lipids (20), and in-house-determined parameters for the cofactors (13). The same setup of MD simulation was used previously for the A-type C*c*O (13). The MD simulations were performed with the software NAMD (21) using a 2 fs time step with SHAKE to fix the bond lengths involving hydrogen atoms and Langevin dynamics with friction constant $\beta = 1\ ps^{-1}$ at 300 K temperature. The MD simulations were performed with a flexible cell size and constant ratio of 1:1 for the *x* and *y* dimensions to stabilize the membrane, which was placed in the *x*-*y* plane. All MD simulations (listed in Table 1) exhibited stable geometries for 100 ns, with root mean-square deviation (RMSD) values for the backbone atoms essentially below 1.2 Å (Fig. S1 in the Supporting Material) and all-atom RMSD values essentially below 1.5 Å (Fig. S2).

Atomic partial charges of C*c*O cofactors

We calculated the atomic partial charges of the cofactors as described by Woelke et al. (13), using the quantum-chemical program Jaguar v.7.7 (Schrödinger, LLC, New York, NY) and the B3LYP DFT functional with the LACVP** basis set. First, the cofactor geometries were optimized quantum chemically. All hydrogen atoms were geometry optimized without constraints, whereas the coordinates of nonhydrogen atoms were optimized with respect to bond lengths and bond angles, keeping the corresponding torsion angles fixed. We then computed the electrostatic potentials in the vicinity of the cofactors based on the electronic wave functions and charges of the nuclei, using the same procedure we employed for geometry optimization. We determined the atomic partial charges from the electrostatic potentials of the cofactors by employing a two-stage restraint-electrostatic-potential (RESP) (22,23) procedure. The only difference between our approach and the procedure described by Woelke et al. (13) is that to compute the atomic partial charges of the Cu_B center, we included Tyr-237 covalently bound to His-233. The coordinates and atomic partial charges are given in Table S2.

Computation of pK_A values and electrostatic energies

We investigated the protonation states in equilibrium of all C*c*O residues based on preliminary pK_A computations (24,25) using just a single structure with optimized hydrogen atom positions based on the crystal structure from *T. thermophilus* with the highest resolution of 1.8 Å (6). The histidine

TABLE 1 MD simulations performed in this work

MD simulation	Redox and protonation state of BNC	Specific protonation in channel
1	Fe(IV)=O/Cu_B(II)-OH/Tyr-237-O^-	all channel residues protonated (except Tyr-237)
2	Fe(IV)=O/Cu_B(II)-OH/Tyr-237-OH	all channel residues protonated
3	Fe(IV)=O/Cu_B(II)-OH/Tyr-237-O^-	deprotonated Tyr-244
4	Fe(IV)=O/Cu_B(II)-OH/Tyr-237-OH	deprotonated Tyr-244
5	Fe(IV)=O/Cu_B(II)-OH/Tyr-237-O^-	deprotonated Glu-15B
6	Fe(IV)=O/Cu_B(II)-OH/Tyr-237-OH	deprotonated Glu-15B

protonations (involving 18 His) are given in Table S1. All other titratable groups (Asp, Glu, Arg, and Lys) are charged, with the following exceptions: the charge-neutral Glu-15B at the entrance of the K-channel analog (discussed in the next section) and the charge-neutral Glu-203, Glu-131B, and Asp-372, which might be involved in proton pumping, but were not analyzed in this study because they are far from the K-channel analog.

To calculate the energy landscape of the proton transfer pathway via the K-channel analog, we used the crystal structure PDB 3S8F (6) embedded in a membrane as described above. The BNC ligands were modeled as described in Woelke et al. (13). We added hydrogen atoms and adjusted the geometry of OH groups of water and side chains (tyrosine and serine) to the different protonation states that potentially can occur during proton transport in the K-channel analog. This modeling also involved adjusting the Tyr-244 side chain for the Tyr-244–Thr-312 switch mechanism (explained in the Results and Discussion section), followed by constrained energy minimization of the modeled region. The pK_A values were calculated using karlsberg+ (24,25). Crystal water molecules were removed (except for the three crystal water molecules inside the K-channel analog (W614, W630, and W689), which were kept) and a dielectric continuum with $\varepsilon = 80$ was placed in the resulting cavities. Lipid molecules of the membrane with atoms within a 20 Å sphere around the center of the K-channel analog were kept. All other lipids were deleted. We tested this procedure and found it to be appropriate (15). A dielectric continuum with $\varepsilon = 80$ was placed outside of the volume of all atoms (from protein, lipids, and water molecules) that were kept explicitly, whereas inside the volume of explicit atoms, $\varepsilon = 4$. The moderate value of the dielectric constant of $\varepsilon = 4$ inside the volume of explicit atoms accounts implicitly for a limited variation of atom positions, which is not considered explicitly when only the crystal structure is used to evaluate electrostatic energies. Since the electrostatic continuum implicitly accounts for conformational variability, the computed electrostatic energies also contain entropy contributions from the protein and solvent.

For each of the titratable residues involved in proton translocation inside the channel (Tyr-237, farnesyl$_{hemea3}$, W614, Ser-309, Tyr-244, Thr-312, Tyr-248, W630, Thr-315, and Glu-15B), a structure was modeled for the corresponding deprotonated state. For each of these structures, the pK_A values and thus the protonation energies of the corresponding residue were calculated with karlsberg+ (24,25). To account for structural changes due to the modeling, the electrostatic contribution to the conformational energy was added to the protonation energy. All of these energy terms were calculated by numerically solving the linearized Poisson-Boltzmann equation using the program APBS (26). To obtain reliable results, the finest grid spacing of 0.25 Å was used.

Role of electrostatic energy in characterizing proton transfer energetics

A precise evaluation of proton transfer energetics requires a consideration of the free energy, which is computationally difficult to assess. Usually, it requires MD simulations beyond the microsecond time regime and is subject to the uncertainties of the employed energy function, which for such long simulation times may drive the molecular system into unphysical conformational regimes. The free energy combines the influence of enthalpy and entropy. Whereas entropy is only indirectly accessible by the conformational variability, enthalpy relies on the interplay between kinetic and potential energies. Based on the second viral theorem (27), the average kinetic energy is proportional to the number of degrees of freedom of the molecular system and therefore does not change in a proton transfer process.

The enthalpy consists of bonded (covalent) and nonbonded (van der Waals (vdW) and Coulomb) energies. A critical part of the bonded energy is the contribution of the transferred proton that is covalently bound to different molecular groups before and after the transfer step. However, we showed that ab initio computations of pK_A values using a combination of high-precision quantum-chemical and electrostatic energy evaluations yield an RMSD to corresponding measured values of 0.5 pH units (28). This agreement demonstrates that the thermodynamic averages of 1), nonelectrostatic quantum-chemical contributions to pK_A values do not depend on the environment; 2), vdW interactions are practically identical before and after a change in protonation; and 3), covalent interactions inside other not directly covalently connected molecular groups (of the solute) that are spectators in the considered proton transfer step also do not change. Further support for these conclusions comes from extensive evaluations of pK_A values of titratable groups in proteins based on the measured pK_A values in solution (24). These pK_A values are transformed by electrostatic energy computations to the corresponding protein environment with an RMSD to measured values of 1.1 pH units. Hence, it is justifiable to consider only electrostatic energies to characterize proton transfer processes, since they make the main contribution to the energetics of such processes.

Another reason to consider only the electrostatic energies is the fact that our computations are based on crystal structures. This may yield arbitrary contributions for covalent and vdW interactions, since these interactions are very sensitive to small variations of 0.1 Å in atomic coordinates for individual conformations, whereas electrostatic energies with their very mild 1/r distance dependence are relatively insensitive to such small changes. Although the electrostatic energies computed in this study refer to a single conformation, they implicitly consider many conformations by using a dielectric continuum for the solvent and the protein. Thus, they also account for entropy contributions as noted above.

RESULTS AND DISCUSSION

Investigation of the K-channel analog

After oxygen splitting occurs, the first proton is likely taken up in the P_R state (29), which is characterized by an access electron inside the BNC that is composed of Fe(IV)=O/Cu(II)-OH/Tyr-237-O^-. The K-channel analog, as depicted in Fig. 1, starts with Glu-15B at the N side of the membrane and terminates at Tyr-237. As has been shown by quantum-chemical calculations (30), the proton wire in the channel is composed of several H-bonded OH groups that are protonated in the resting state and conduct protons via a proton-hole transfer mechanism. We performed MD simulations to investigate the conformational stability or mobility of residues in the K-channel analog in different protonation states. A 100 ns MD simulation was performed with all three subunits of C*c*O embedded in a phospholipid membrane, as described in Materials and Methods, with the BNC modeled in the P_R state (Table 1, simulation No. 1). In the P_R state (Table 1, No. 1), the K-channel analog exhibits a very stable geometry for the full MD simulation time, as evidenced, for example, by the low RMSD value of 0.8 Å of the K-channel analog residues (alignment on all backbone atoms and RMSD measurement on all atoms of channel residues; Fig. S3). All H-bonds from Glu-15B to the deprotonated Tyr-237 are pointing toward the BNC fluctuating around an oxygen-oxygen distance of 2.8 Å corresponding to ideal H-bond geometry (Fig. S4 *A*). Tyr-244 donates an H-bond to Ser-309, whereas Thr-312 donates its H-bond to the backbone oxygen of Pro-308 (see Fig. 1), thus leaving a gap of ~5 Å in the H-bond chain, which is not bridged by a water molecule throughout the simulation (see further discussion below). The crystal water molecule W614 moves from

downward of the Tyr-237 oxygen to upward, but remains H-bonded to the negatively charged Tyr-237 side chain. The other two crystal waters inside the K-channel analog, W630 and W689, remain at their positions during the whole MD simulation time of 100 ns. This rigid channel geometry may be essential for function in the high-temperature environment in which *T. thermophilus* resides.

The MD simulation with protonated Tyr-237 (Table 1, No. 2) exhibits a geometry similar to that observed in the simulation with deprotonated Tyr-237, but some H-bonds of the K-channel analog are slightly less stable (see Fig. S4 *B*). Tyr-237 loses its H-bond contact to the OH group of farnesyl from heme a_3, and also the H-bond between Ser-309 and Tyr-244 is split, but bridged by a water molecule.

The proton-hole sink: Glu-15B

The entrance of the K-channel analog consists of the glutamate residue Glu-15B, which is conserved among channels of A- and B-type C*c*O (5,6,31,32). We performed MD simulations with protonated (Table 1, No. 2) and deprotonated (Table 1, No. 6) Glu-15B, and found that the protonated Glu-15B forms a stable H-bond toward Thr-315 with an average distance of 2.8 Å (Fig. S4, *A* and *B*) over 100 ns (Fig. 2 *A*), whereas the deprotonated Glu-15B does not (Fig. S5, *A* and *B*). The deprotonated Glu-15B first accepts an H-bond from Thr-315 (Fig. 2 *B*), but loses this contact after 10.8 ns or 1.4 ns MD simulation with protonated Tyr-237 or deprotonated Tyr-237, respectively. The deprotonated Glu-15B swings out of the hydrophobic channel entrance toward bulk water, as has also been demonstrated by quantum-chemical calculations (30,33), and does not return in the deprotonated state (Fig. S5, *A* and *B*). As soon as Glu-15B loses contact to Thr-315, the H-bonds of the proton wire of the K-channel analog (see Fig. 1) turn inward, pointing with the hydrogen atoms toward the BNC (Fig. 2 *C*). After a >40 ns simulation with deprotonated Glu-15B, the water molecule W630 leaves the channel toward bulk water without returning (Fig. S5, *A* and *B*). This creates a gap between Thr-315 and Tyr-248, which slightly destabilizes the H-bond chain in the K-channel analog.

In the crystal structure PDB 3S8F (6), Glu-15B is pointing toward Thr-315 (see Fig. 1) and may either be protonated and donate an H-bond to Thr-315 or be deprotonated and accept an H-bond. Preliminary pK_A computations based solely on the crystal structure yield a pK_A value for Glu-15B of 6.8. However, in MD simulations, the deprotonated Glu-15B moves out of the K-channel analog toward bulk water and the protonated Glu-15B remains precisely at the crystal structure position. Thus, we conclude that Glu-15B is protonated in the 3S8F structure (6). Interestingly, the other crystal structures of C*c*O from *T. thermophilus* exhibit a Glu-15B position more distant from Thr-315 (3.41 Å in PDB 1EHK (34) and 3.64 Å in PDB 1XME (35)) and the mean B factor of the Glu-15B side chain atoms is 41.8 $Å^2$ larger than the mean B factor (26.1 $Å^2$) of the side-chain atoms of the other K-channel analog residues in the crystal structure 3S8F (6) (similar relations hold for the other crystal structures). This suggests that Glu-15B is more flexible and may occasionally move from its main conformation, where it is protonated and points inward to a second conformation that is deprotonated and points to bulk water.

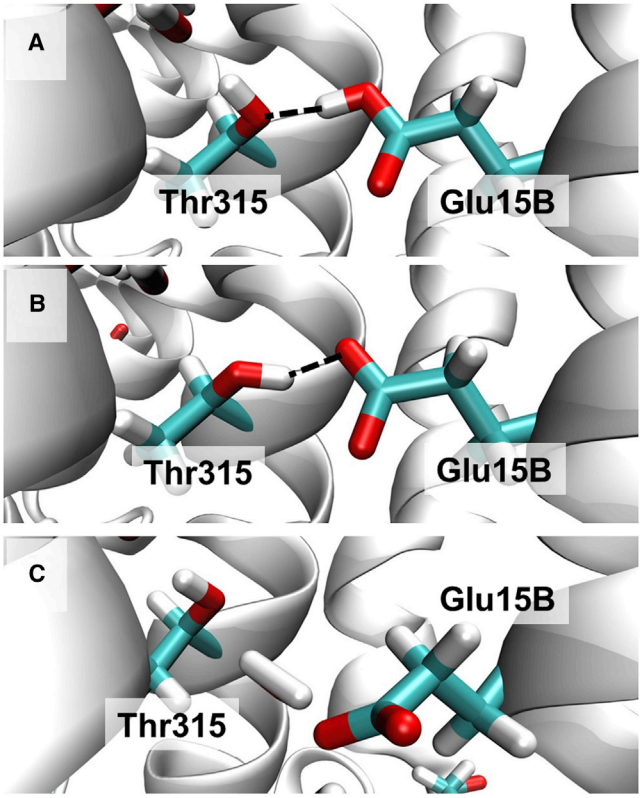

FIGURE 2 Different positions of Glu-15B depending on the protonation state. (*A*) The crystal structure (PDB ID: 3S8F (6)) with protonated Glu-15B donating an H-bond to Thr-315. The MD simulation with protonated Glu-15B remains close to this position. (*B*) After energy minimization, deprotonated Glu-15B accepts an H-bond from Thr-315. (*C*) Snapshot of the MD simulation after 15.0 ns. Glu-15B moves out of the K-channel analog toward bulk water and loses connectivity to Thr-315, which then forms an H-bond inward with the hydrogen atom pointing to the BNC and remains there for the rest of the 100 ns MD simulation. H-bonds are indicated by dashed lines. To see this figure in color, go online.

In summary, Glu-15B of the K-channel analog is likely protonated and donates an H-bond to Thr-315 in the resting state of the channel, where C*c*O is ready to take up a new electron. However, Glu-15B may easily donate its proton, become deprotonated, and move out toward the channel entrance in bulk water, where it may transiently accept a new proton. This behavior of Glu-15B renders it an excellent sink for proton holes, promoting rapid proton uptake by C*c*O.

Tyr-244, the valve

In the crystal structure obtained by Tiefenbrunn et al. (6), the only gap in the H-bond chain of the K-channel analog

is between Thr-312 and Ser-309, which are 4.9 Å apart (oxygen-oxygen distance; Fig. 1). This gap could be bridged either by Tyr-244 swinging toward Thr-312 or by one additional water molecule, as suggested previously (11). However, after carefully inspecting the latest crystal structure at 1.8 Å resolution, Tiefenbrunn et al. (6) did not observe an electron density that could correspond to a water molecule bridging this gap. Furthermore, the Tyr-244 position is virtually identical in all available crystal structures and has a low B factor throughout (6,34,35). Likewise, all of our modeling attempts to move Tyr-244 or place an additional water molecule inside this gap did not lead to a stable conformation in MD simulations (data not shown). Instead, we observed a switching mechanism of Tyr-244.

Whereas the protonated Tyr-244 remained at the crystal structure position during the whole 100 ns MD simulation (Fig. 3 *A*; Table 1, No. 2; H-bond distances in Fig. S6, *A* and *B*), the deprotonated Tyr-244 immediately moved between Ser-309 and Thr-312, accepting H-bonds from both of them (Fig. 3 *B*; Table 1, No. 3; H-bond distances in Fig. S6, *A* and *B*). This geometry was stable for only 0.3 ns, and then a water molecule that was placed inside the protein during solvation moved between Tyr-244 and Ser-309, such that they were still H-bond connected (Fig. and H-bond distances in Fig. S6, *A* and *B*). The other H-bond between Thr-312 and Tyr-244 remained stable for practically the whole 100 ns simulation, with an average distance of 2.8 Å (Fig. 3 C and H-bond distances in Fig. S6, *A* and *B*).

In conclusion, the gap between Ser-309 and Thr-312 is only bridged by Tyr-244 after its deprotonation. This behavior renders the Tyr-244–Thr-312 pair an optimal switch, ensuring unidirectional proton transport. In the resting state of the channel, where C*c*O is ready to take up a new electron, Tyr-244 is only H-bonded with the upstream Ser-309 close to the BNC, whereas Thr-312 donates an H-bond to the Pro-308 backbone oxygen atom, which has no hydrogen atom that could potentially leak toward the channel entrance. Only after Tyr-244 has donated its proton toward the BNC does the Tyr-244–Thr-312 switch make the K-channel analog proton conducting again. The deprotonated Tyr-244 may quickly obtain a new proton from Glu-15B at the channel entrance, which converts the protonation of the K-channel analog back to the resting state of the channel. Thus, the K-channel analog does not function solely via a simple Grotthuss proton conductor consisting of a single wire of connected H-bonds (36), as suggested previously (11,30); rather, it is a two-wire Grotthuss conductor interrupted by Tyr-244, which if deprotonated makes a conformational change to connect the two wires transiently.

We also calculated the so-called action pK_A of Tyr-244, assuming that a (nonequilibrium) proton hole is localized at Tyr-244. Such action pK_A values describe the local electrostatic energies within the reaction sequence of proton-hole transfer inside the K-channel analog. The alternative pK_A of Tyr-244 in equilibrium with solvent pH is not useful for our question of interest because it depends on the changes in protonation pattern of all other titratable residues and the corresponding conformational change of the protein. However, these changes do not reflect the physiological conditions of the proton-transfer reaction. The action pK_A for Tyr-244 is 7.0 in a conformation corresponding to the crystal structure involving only one H-bond with Ser-309. However, this value drops to 2.8 with the conformational change of Tyr-244 accepting two H-bonds from Ser-309 and Thr-312. This clearly demonstrates that the deprotonated Tyr-244 needs to be stabilized by an additional H-bond from Thr-312, whereas the protonated Tyr-244 is in a more stable conformation when it donates only one H-bond to Ser-309, as observed in the crystal structure.

The Tyr-244–Thr-312 switch is supported by experimental data. The Thr312Val mutant was shown to have more severe effects on C*c*O function than other channel mutants, such as Tyr248Phe and Thr315Val, which strongly reduced or even abolished enzymatic activity in experiments (11,37,38). In a study by Smirnova et al. (39), the Thr312Val and Tyr244Phe mutants of C*c*O were able to take up the first proton after oxygen splitting, but the efficiency of the second proton uptake was significantly reduced. The authors concluded that these mutations interfere with structural changes that are rate limiting for the second proton transfer (39). In this study, we have demonstrated that the suggested structural change is connected to the ability of Tyr-244 to

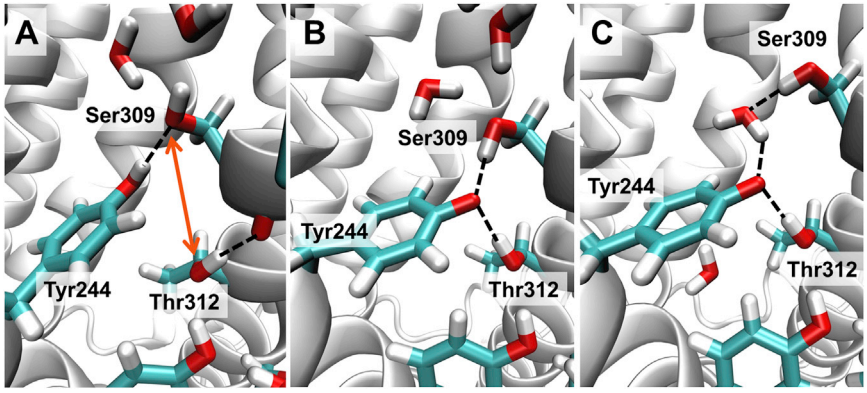

FIGURE 3 Different positions of Tyr-244 depending on its protonation state. (*A*) The crystal structure (PDB ID: 3S8F (6)) with protonated Tyr-244. The 4.9 Å gap between Thr-312 and Ser-309 is indicated by an orange arrow. During the MD simulation with Tyr-244 protonated (Table 1, No. 2), it remains close to the position in the crystal structure. (*B*) Snapshot of the MD simulation with deprotonated Tyr-244 (Table 1, No. 4) after 50 ps. The deprotonated Tyr-244 moves into the Thr-312-Ser-309 gap immediately. H-bonds are indicated by dashed lines. (*C*) Snapshot of the MD simulation with deprotonated Tyr-244 (Table 1, No. 4) after 17.6 ns. One water molecule moves in between Ser-309 and Tyr-244.

move and fulfill its switching function in the K-channel analog, thereby controlling proton conductance.

Tyr-237: the branching point

The K-channel analog terminates at Tyr-237, which is covalently bound to one of the Cu_B-ligating histidines and is conserved in all types of C*c*O (4–6). It takes part in the enzymatic reaction of C*c*O by donating a proton as well as an electron in the oxygen-splitting reaction, thus forming a tyrosyl radical. In the catalytic cycle, this Tyr-237 radical receives a new electron and a new proton in separate steps. In this study, we investigated the P_R state, in which Tyr-237 has received an electron but not yet a proton. Interestingly, the F state could not be observed spectroscopically in the B-type C*c*O (29,40), perhaps because in the B-type C*c*O, the Tyr-237 receives the first chemical proton (41) instead of the Cu_B-ligating hydroxyl as in A-type C*c*O. The state after this proton is received in the B-type C*c*O may be the F state or an intermediate before F-state formation.

We also performed MD simulations with protonated (Table 1, No. 2) and deprotonated (Table 1, No. 1) Tyr-237 (Fig. 4). In both MD simulations, the H-bond network of the K-channel analog is very stable. For deprotonated Tyr-237 (Table 1, No. 1), all H-bonds of the K-channel analog point inward to the BNC, whereas the crystal water W614 moves slightly above Tyr-237 but remains H-bonded with it (H-bond distances in Fig. S4 *A*). For the protonated Tyr-237 (Table 1, No. 2), the H-bonds of the proton wire in the upper part of the channel point down toward the N side, but in the lower part of the channel from Thr-312 to Glu-15B, the H-bonds still point inward to the BNC (H-bond distances in Fig. S4 *B*). This underlines the function of the Tyr-244–Thr-312 switch, because this geometry is not prone to proton leakage from Tyr-237 to the N side of the K-channel analog.

The oxygen atom of Tyr-237 is located at a distance of 5.0 Å from the oxygen ligand of heme a_3 (6) (Fig. 4) and may donate chemical protons to this oxygen, probably via one or two water molecules (a suggested pathway is indicated in Fig. 4), which are not present in the crystal structure and thus may occupy their positions only transiently. Tyr-237 is the residue that terminates the proton input channel at the BNC. Since there is only one proton input channel in B-type C*c*O, it is suggestive that also the pumped protons are transferred via Tyr-237. No alternative pathway for proton transfer from the K-channel analog to the putative proton loading site (PLS) is visible in the C*c*O crystal structure (Fig. 4). Between Tyr-237 and the putative PLS, which was proposed to be around the propionates of heme a_3 (42,43), the only titratable groups are the Cu_B ligands and Thr-302 (Fig. 4). The surroundings of Tyr-237 involve several hydrophobic residues, which are not suitable for proton transfer. Therefore, Tyr-237 may likely be the branching point at which the routes of chemical and pumped protons diverge (suggested pathways are indicated in Fig. 4). A function analog to Tyr-237 is accomplished by Glu-286 in the D-channel of A-type C*c*O (2) (*R. sphaeroides* numbering).

The approximate location of the pathways for chemical and pumped protons beyond Tyr-237 is indicated in Fig. 4. However, the mechanism underlying how chemical and pumped protons are differentiated beyond Tyr-237 needs further investigation. Proton movement is controlled by electrons entering the BNC and, most likely, chemical protons are dragged from Tyr-237 into the BNC by a low pK_A of the oxygen ligand at heme a_3 and pumped protons are dragged by a low pK_A of the PLS. From the crystal structure with modeled hydrogens, we computed a pK_A of 12.8 for Tyr-237 in the P_R state based on electrostatic calculations. However, as Tyr-237 is covalently bound to the Cu_B-ligating histidine, its pK_A will be strongly influenced by the redox-state of Cu_B and refinement of the pK_A computation using quantum-mechanical calculations will be needed.

The actual protonation state of Tyr-237 in the crystal structure (PDB ID: 3S8F (6)) cannot be determined with

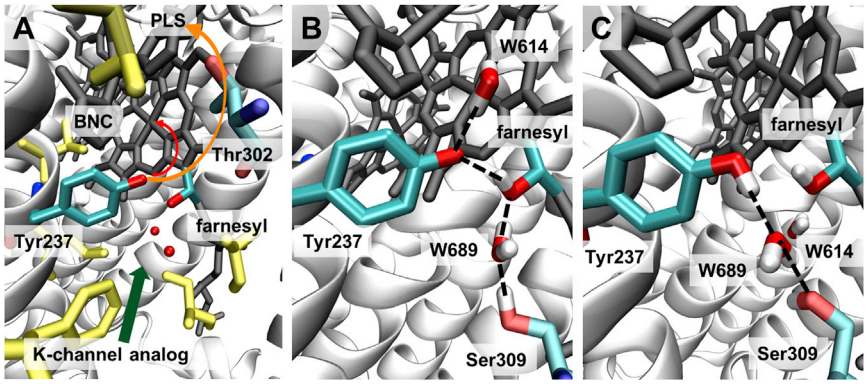

FIGURE 4 Different positions of Tyr-237 depending on its protonation state. (*A*) The crystal structure (PDB ID: 3S8F (6)) with the environment of Tyr-237. Tyr-237 at the end of the K-channel analog (*green arrow*) is close to or even part of the BNC (*black stick model*) and at a 5.0 Å distance from the OH group of farnesyl from heme a_3. The putative PLS is above the BNC, with a few hydrophilic residues (e.g., Thr-302) in between. Hydrophobic residues around Tyr-237 are shown in yellow. The putative pathways of the chemical (*red*) and pumped (*orange*) protons above Tyr-237 are indicated by arrows, but the exact locations of the proton pathways are not known. (*B*) Typical snapshot of MD simulation with deprotonated Tyr-237 (Table 1, No. 1) at 47.0 ns. The deprotonated Tyr-237 remains at the crystal structure position with a stable H-bond from farnesyl of heme a_3 (O-O distance 2.6 Å). The crystal water W614 moves above Tyr-237 and forms a stable H-bond (O-O distance 2.8 Å). H-bonds are indicated by dashed lines. Nonpolar hydrogens are not shown. (*C*) Typical snapshot of MD simulation with protonated Tyr-237 (Table 1, No. 2) at 64.0 ns. The H-bond between Tyr-237 and farnesyl of heme a_3 is lost, and the water molecule W614 bridges Tyr-237 and Ser-309 (the O-O distances of Tyr-237 and Ser-309 with W614 are 2.8 Å and 2.6 Å, respectively).

certainty. Still, two arguments point to a protonated Tyr-237: 1), the average geometry from MD simulation with a protonated Tyr-237 deviates the least from the crystal structure geometry, and even crystal water molecules around Tyr-237 remain at their position; and 2), if Tiefenbrunn et al.'s (6) conclusion about a peroxide in the BNC is correct, Tyr-237 would be in the state right before donating a proton.

Proton transfer energy landscape

To demonstrate how the K-channel analog might operate, we calculated the electrostatic contribution of the energy differences of all steps of proton transport using our software karlsberg+ (24,25). However, these energy barriers are only approximate because we used the crystal structure (6) for our calculations and adjusted only the BNC ligands to the corresponding redox state with split dioxygen (see Table 1), added hydrogen atoms, made small geometric adjustments of the OH groups for the different protonation states, and modeled the conformational change of Tyr-244 for the Thr-312–Tyr-244 switch. The proton-transfer sequence starts in the P_R state with a deprotonated Tyr-237 and all other residues involved in K-channel proton transport in their neutral-charge state. As can be seen in Fig. 5, almost all proton-transfer steps exhibit a favorable electrostatic energy relative to the proton hole at the starting point at Tyr-237. The most unfavorable energy is observed for the crystal water W630 forming a hydroxyl ion, which is due to the high general pK_A value of water in solution ($pK_A = 15.75$). However, another reason for the large activation barrier could be the uncertainty of the oxygen atom position of W630, which is taken from the C*c*O crystal structure and may differ under physiological conditions in the proton-transfer reaction.

Our results demonstrate the feasibility of the Tyr-244–Thr-312 switch, since deprotonation of Tyr-244 is energetically favorable when it accepts one H-bond from Ser-309, but becomes even more favorable when Tyr-244 changes its conformation to form H-bonds with both Ser-309 and Thr-312 (Fig. 5).

In the last step in proton-hole conduction in the K-channel analog, Glu-15B donates its proton with the largest favorable energy. This may ensure that no negative charge accumulates inside the proton channel that could lead to proton leakage through the channel. The deprotonated Glu-15B then swings out toward bulk water and receives a new proton to fulfill its function to serve as proton-hole sink at the channel entrance.

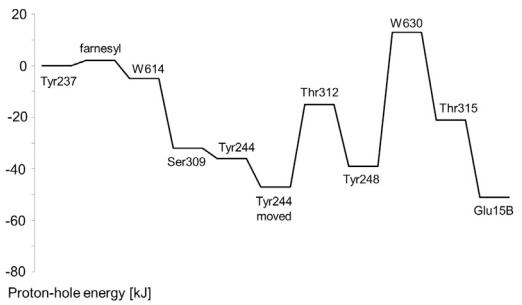

FIGURE 5 Electrostatic energy landscape of the proton hole inside the K-channel analog. Electrostatic energies for all deprotonation steps were calculated using the software karlsberg+ (24,25) as described in Materials and Methods. Transport of a proton hole along the K-channel analog starts at Tyr-237, which is deprotonated and donates its proton to the BNC. The proton hole subsequently moves along a linear chain of deprotonatable residues until it reaches Glu-15B. After deprotonation of Tyr-244, this residue moves inside the gap between Ser-309 and Thr-312. The electrostatic energies before and after this conformational change are depicted.

CONCLUSIONS

In this study, we analyzed the properties of the K-channel analog in C*c*O of *T. thermophilus*. We demonstrated that proton transport via the K-channel analog works smoothly, with no oversized energy barriers, using a proton-hole transport mechanism. We identified two important functionalities: 1), Glu-15B at the channel entrance is a proton-hole sink; and 2), unidirectional proton flow is accomplished by the Tyr-244–Thr-312 switch, where deprotonated Tyr-244 moves inside the gap between Thr-312 and Ser-309. These findings may explain the outcome of several mutational studies and may inform the setup for new experiments. Furthermore, enhanced knowledge about the K-channel analog will provide an important basis for investigating the aberrant pumping properties of B-type as compared with A-type C*c*O (44).

SUPPORTING MATERIAL

Six figures, two tables, RMSD values for all simulations, important hydrogen-bond distances, partial charges of cofactors, and the histidine protonation pattern are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(14)00948-5.

The authors thank Dr. Joachim Heberle for fruitful discussions.

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Supporting Information

Proton Transfer in the K-Channel Analogue of B-Type Cytochrome *c* Oxidase from *Thermus thermophilus*

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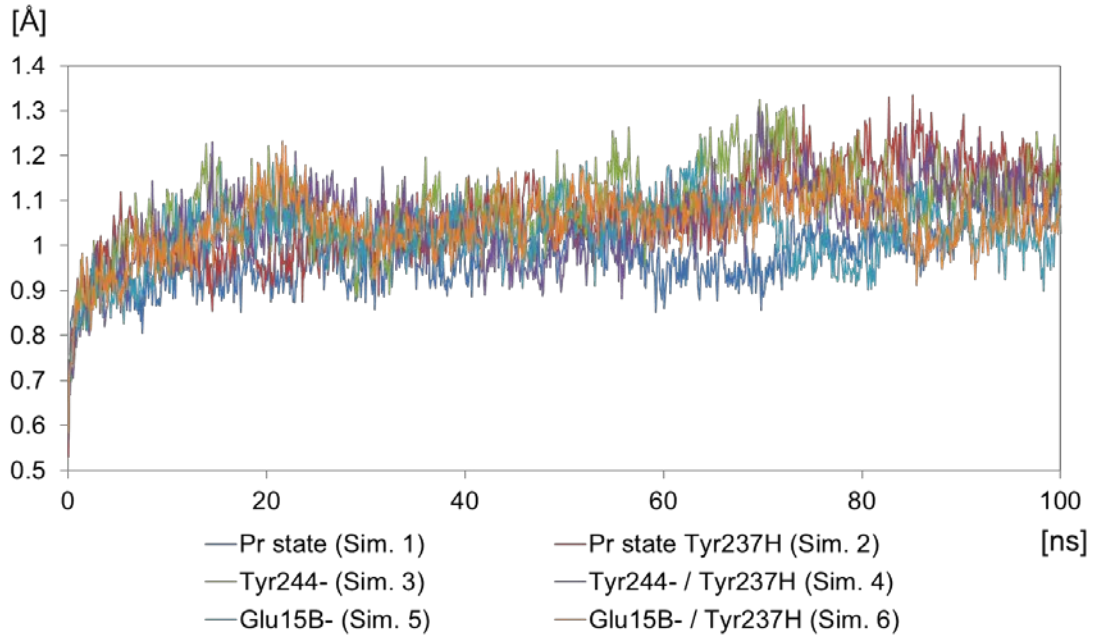


Figure S1: Root mean square deviation (RMSD) of C*c*O backbone atoms relative to the crystal structure PDB-id 3S8F [2] obtained by MD simulations for all simulations performed (for simulation details see Table I in main manuscript).

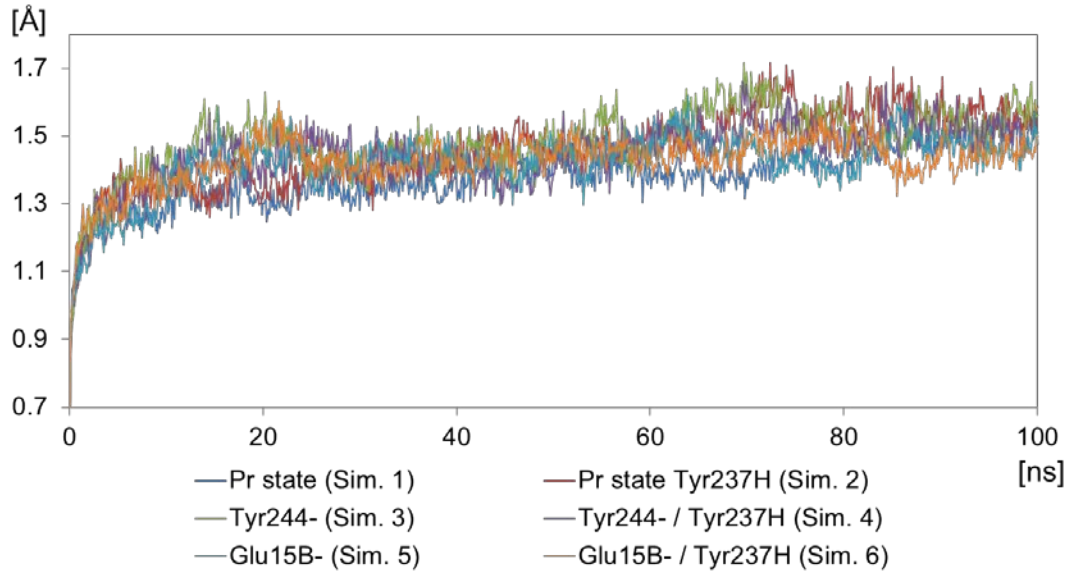


Figure S2: Root mean square deviation (RMSD) of C*c*O of all protein atoms relative to the crystal structure PDB-id 3S8F [2] obtained by MD simulations for all simulations performed (for simulation details see Table I in main manuscript).

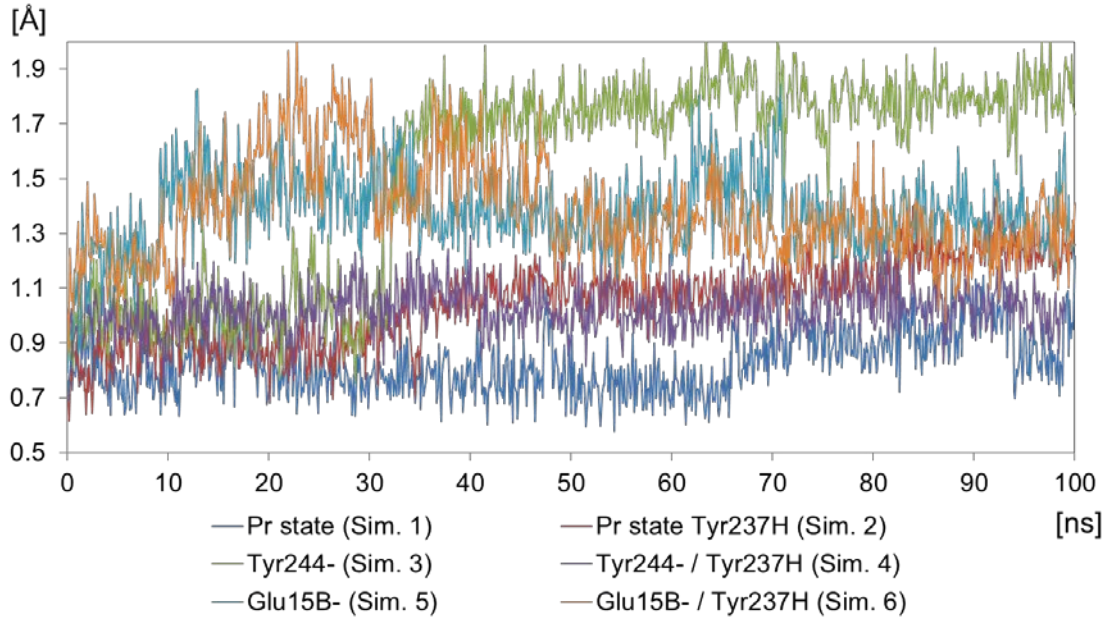


Figure S3: Root mean square deviation (RMSD) of C*c*O of K-channel analogue residues. MD simulation frames were aligned on the backbone atoms of the crystal structure (PDB-id 3S8F [2]). Then, RMSDs were measured for all atoms of the K-channel analogue residues (Tyr237, Ser309, Tyr244, Thr312, Tyr248, Thr315, Glu15B).

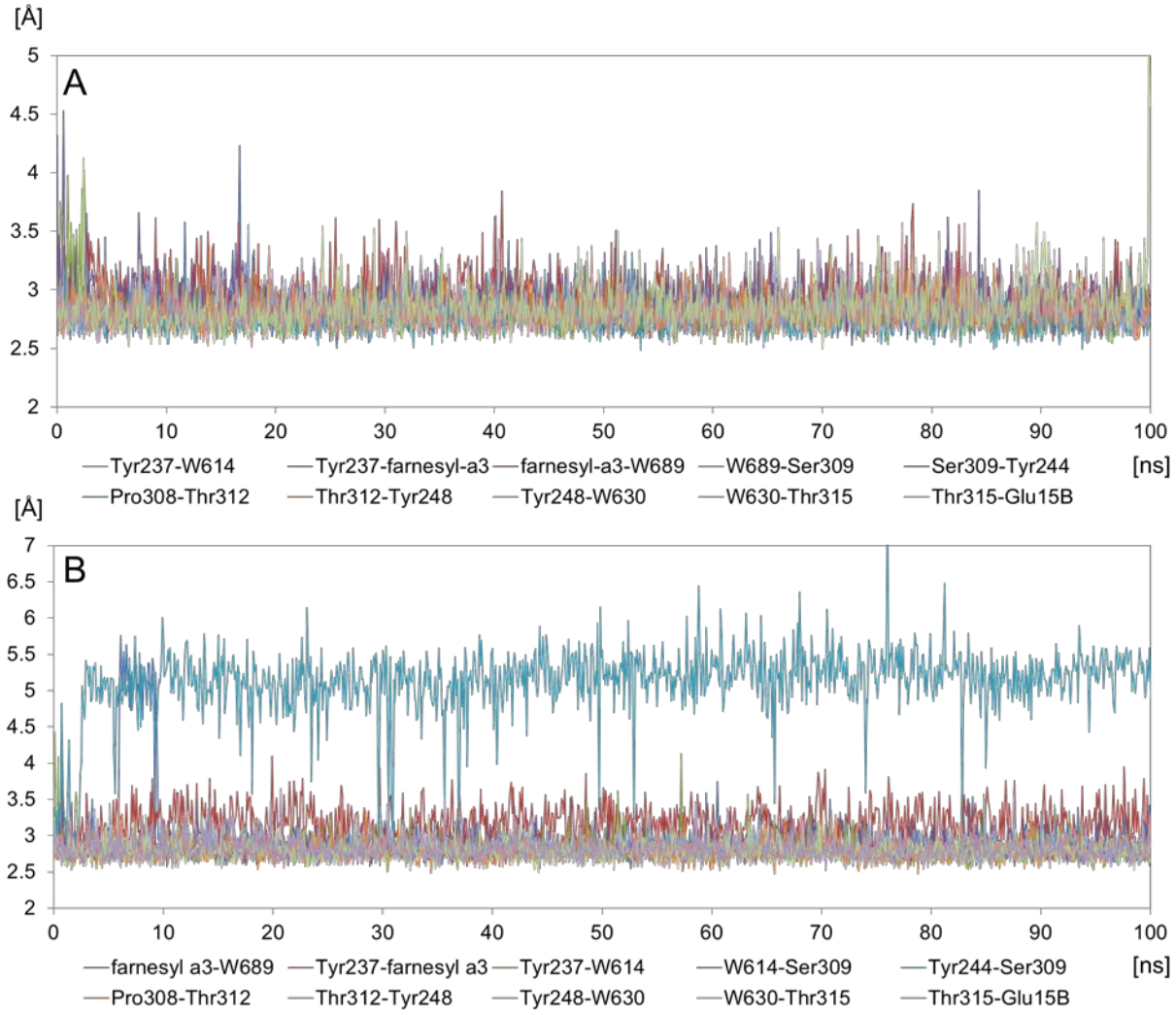


Figure S4: Hydrogen-bonding distances between polar oxygen atoms in the K-channel analogue. Distances between oxygen atoms were recorded for the 100 ns MD trajectory in (A) P_R state and (B) with Tyr237H (for simulation details see Table I, no. 1+2 in main manuscript).

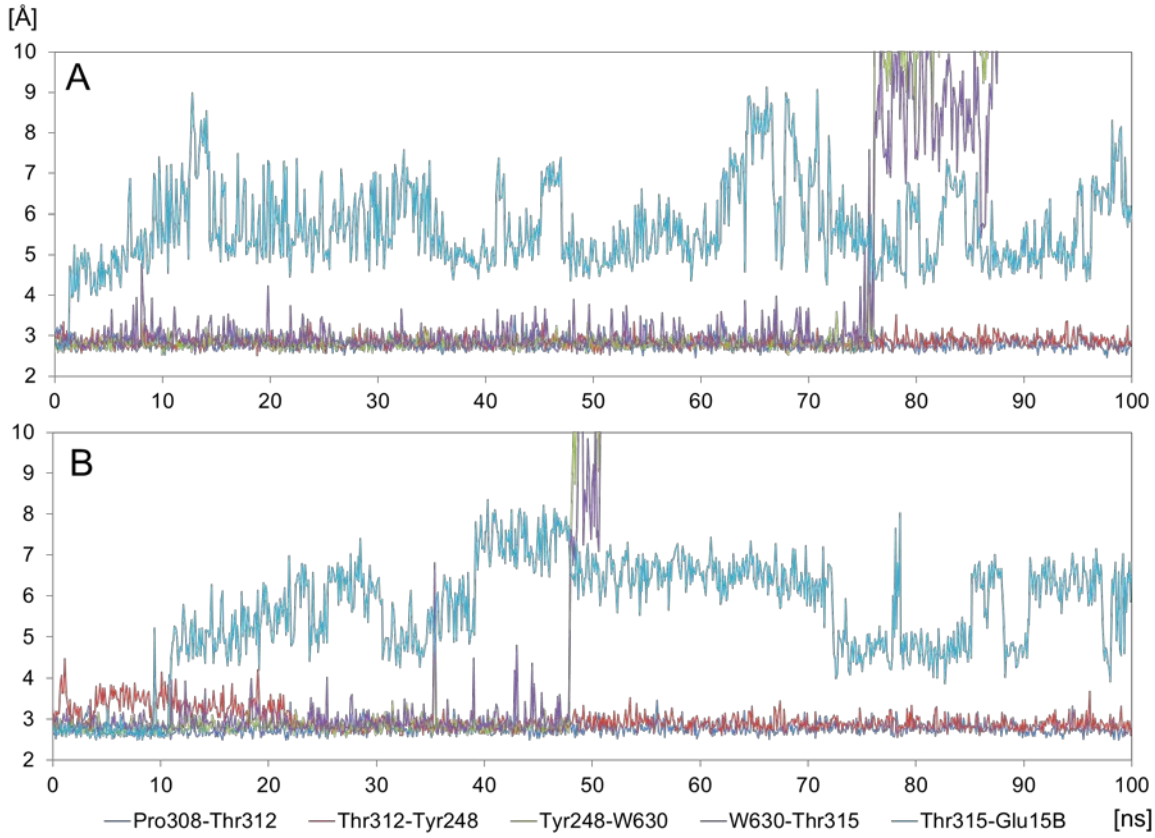


Figure S5: Hydrogen-bonding distances between polar oxygen atoms at the entrance of the K-channel analogue. Distances between oxygen atoms were recorded for the 100 ns MD trajectory with deprotonated Glu15B in (A) P_R state and (B) with Tyr237H (for simulation details see Table I, no. 5+6 in main manuscript).

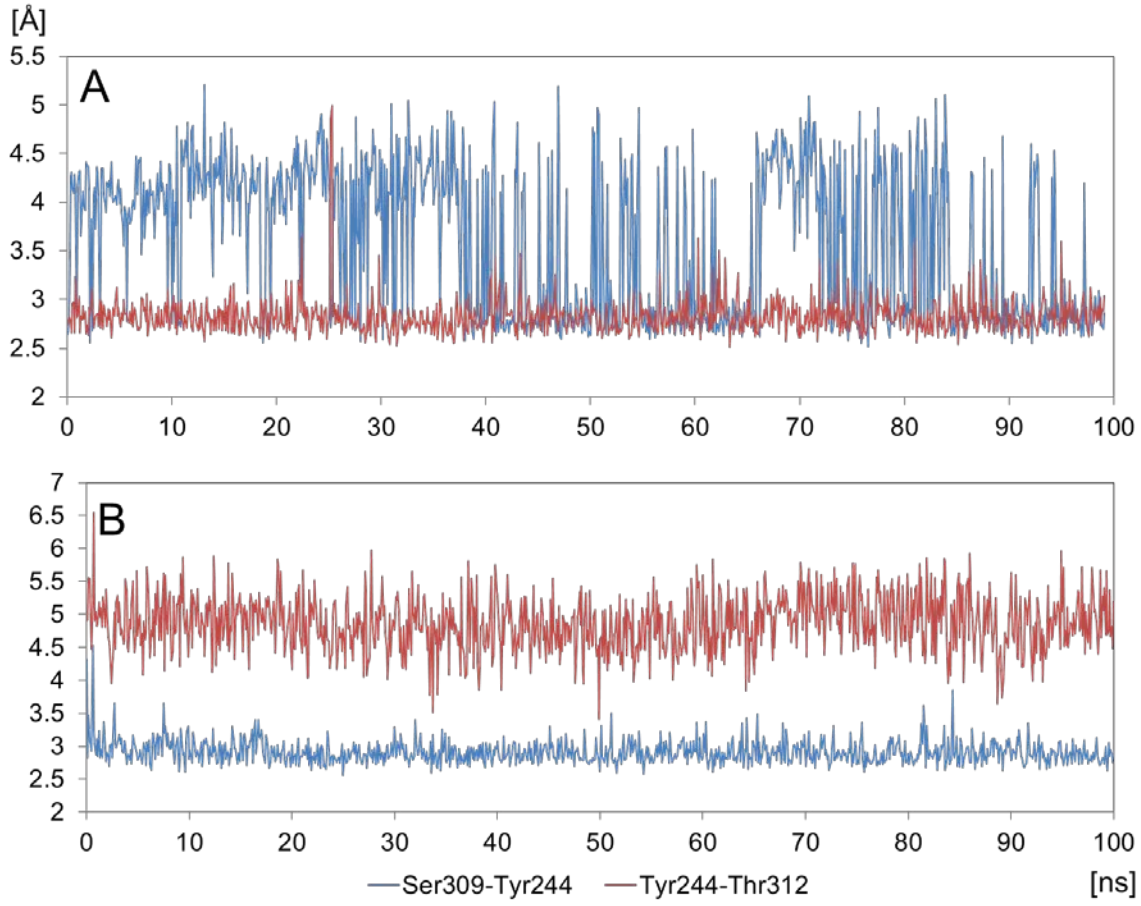
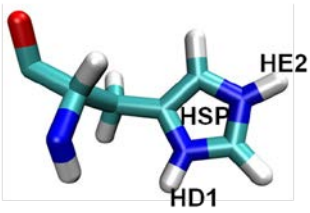
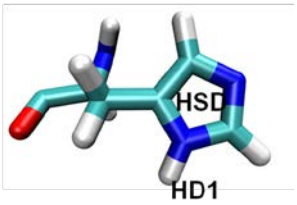
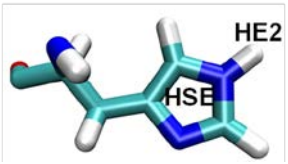


Figure S6: Hydrogen-bonding distances between polar oxygen atoms in the middle of the K-channel analogue. Distances between oxygen atoms were recorded for the 100 ns MD trajectory with (A) deprotonated Tyr244 and protonated Tyr237 and (B) in P_R state (for simulation details see Table I, no. 3+1 in main manuscript).

Table S1: Protonation states of the histidines at pH=7 in C*c*O (PDB-id 2GSM).







The different protonation states of histidine are called HSP, HSD and HSE in the CHARMM[1] program.

AA	72 (A)	142 (A)	233 (A)	282 (A)	283 (A)	298 (A)	376 (A)	384 (A)	386 (A)
HisType	HSD	HSE	HSE	HSD	HSD	HSE	HSP	HSD	HSD

440 (A)	462 (A)	552 (A)	5 (B)	8 (B)	40 (B)	114 (B)	117 (B)	157 (B)
HSD	HSE	HSD	HSE	HSD	HSE	HSE	HSE	HSE

Table S2: Geometry-optimized coordinates and atomic charges for the BNC cofactors.

a) CuA in oxidized state (used for all calculations)

Cu1	-30.3270214596	148.9184511061	13.7615419482	0.311997
Cu2	-28.4194902574	147.0332691630	14.5574729498	0.311997
H3	-25.2084464886	148.7142486770	14.3672519247	0.132877
N4	-26.4180447344	147.0350061074	13.7613730923	-0.213947
C5	-25.2792104960	147.8039855783	13.7948458658	0.024317
C6	-26.3104004498	145.9874156863	13.0149433953	0.097385
H7	-27.0472576587	145.2276439342	12.8076710978	0.104494
N8	-25.0273624388	146.0202440434	12.5116533800	-0.193361
C9	-24.3662249887	147.1531184579	12.9921076707	-0.208905
H10	-23.3478442625	147.3702223383	12.7093699160	0.196572
C11	-30.6264341984	149.2368093769	16.9497028022	-0.005121
H12	-30.4054167205	148.7254743565	17.8889034834	0.051160
H13	-31.5650897477	148.8611676073	16.5406165089	0.051160
S14	-29.2290393107	148.9763774079	15.7792855141	-0.310848
N15	-29.0538688961	152.3462818304	14.3272468683	-0.905063
H16	-29.1249085687	151.3658659433	14.5726316043	0.331005
C17	-28.6592991126	152.5455030526	12.9512767613	0.210796
H18	-28.7145301876	153.6090198907	12.6975313624	0.052321
H19	-27.6134786516	152.2410036342	12.8189304878	0.028775
C20	-29.5141552252	151.6904314385	11.9991776517	0.415545
O21	-29.5435779802	150.4575644807	12.1093223297	-0.425493
N22	-30.4223645331	152.3424376739	11.2262762183	-0.713060
H23	-30.3115998988	153.3212532065	11.0056001449	0.340823
H24	-30.9970633615	151.7859976103	10.6064085380	0.378953
C25	-28.7727473311	147.3833845238	11.2819231234	0.101646
H26	-28.3851671725	146.5076054906	10.7559089054	0.022120
H27	-27.9553689119	148.0159583505	11.6245714914	0.022120
S28	-29.8141884519	146.8154649626	12.6947431327	-0.352773
H29	-33.2808170799	147.8994134365	12.8801623517	0.131131
N30	-32.2897306953	149.6188265009	13.7066361207	-0.147998
C31	-33.3333857278	148.9529431160	13.1069528429	-0.031274
C32	-32.6434209137	150.9186094890	13.8600279503	-0.018683
H33	-31.9863883607	151.7192103060	14.1609385453	0.133179
N34	-33.8670035199	151.0708698156	13.3622134927	-0.158875
C35	-34.3319449733	149.8509296044	12.8869753737	-0.154431
H36	-35.3179097335	149.7535293719	12.4594951925	0.177053
C37	-28.8008645918	144.5433665465	17.5359531210	-0.094367
H38	-29.8338392323	144.8572342928	17.3734838364	0.092250
H39	-28.5264599668	144.7327389645	18.5758743440	0.092250
S40	-27.7683885608	145.5455451624	16.4155875678	-0.220580
C41	-26.0783119079	145.0085812476	16.8369832484	-0.222799
H42	-25.4015144887	145.6254723219	16.2425678160	0.123502
H43	-25.8812513278	145.1873340764	17.8960677150	0.123502
H44	-25.9205965198	143.9567008975	16.5879251985	0.123502
H45	-24.6123510469	145.3410336601	11.8886099439	0.319269
H46	-34.4022407918	151.9283429563	13.3556921216	0.320702
H47	-29.7656802426	152.9419634016	14.7208235833	0.389642
H48	-30.6985977966	150.3070776053	17.1522811330	0.051160
H49	-29.3996092214	147.9516399790	10.5936179505	0.022120
H50	-28.7102417113	143.4791274433	17.3070069628	0.092250

b) heme a in oxidized state (used for all calculations)

H1	1.0239320577	-1.3183000967	38.9398941612	0.181210
C2	1.4909700298	-2.1324224093	38.4082267686	-0.126252
N3	2.4668918501	-2.9237006327	39.0000844501	-0.204047
C4	1.2861378809	-2.6540293935	37.1656361891	-0.136193
C5	2.8746383679	-3.8623337411	38.1188188228	-0.004414
N6	2.1887508740	-3.7038229060	37.0079671504	0.023538
H7	3.3010617514	-7.6716097547	31.0262020711	0.183593
C8	2.8189386081	-7.0847620378	31.7920567270	-0.118088
N9	1.4768473618	-6.7487184046	31.7139378525	-0.212660
C10	3.2757388779	-6.5531276053	32.9675072483	-0.203911
C11	1.1211226057	-6.0245334916	32.7849481888	-0.007644
N12	2.1757196738	-5.8833510427	33.5439998740	0.091744
C13	-1.2033153115	-3.8952969679	34.7482843924	-0.333782
C14	1.0683498935	-7.7360338829	36.8568703076	-0.215239
C15	5.5237873650	-5.7221106855	36.2039619395	-0.212895

C16	3.2880141988	-2.3070323447	33.4493402981	-0.167627
C17	-0.8764851168	-5.1961099437	35.3457753388	0.199004
C18	-1.8233223516	-6.2856272242	35.4378607023	-0.327948
C19	-1.2429350809	-7.2886238244	36.0795785368	0.222301
C20	0.1202440891	-6.9210944822	36.2681782075	0.065534
C21	-1.8264598055	-8.6265806947	36.4280023243	-0.360348
H22	-2.8629855560	-6.2001035017	35.1433657427	0.178094
C23	2.4403890906	-7.4095341963	36.9145825438	0.122940
C24	3.4545716847	-8.2428732404	37.5502690519	0.057027
C25	4.6855252720	-7.6285874638	37.4710702589	-0.005936
C26	4.4177819827	-6.4252747661	36.6297624112	0.096625
C27	3.1206281311	-9.4520075595	38.3799785215	-0.295983
C28	5.9908924779	-8.0940867938	37.9343020613	-0.101349
C29	6.2508615605	-8.6390349075	39.1317923800	-0.319434
C30	5.2927391525	-4.6809894736	35.1270879572	0.028820
C31	6.1902587286	-3.7680275644	34.8045987395	0.102032
C32	5.6135142001	-2.9276240257	33.7719798447	0.054588
C33	4.2854766480	-3.1099013493	34.0684863600	0.039909
C34	7.6814172627	-3.9478418006	34.9632157130	-0.302515
C35	6.1880366110	-1.8085038431	33.0423748568	-0.137651
C36	7.4852979481	-1.5995773340	32.7586685527	-0.301693
C37	1.9841093651	-2.4706336464	33.6606378982	0.134036
C38	0.9696371805	-1.4641848169	33.2148692074	0.086746
C39	-0.2914101054	-1.9201908563	33.6367409301	-0.324167
C40	-0.0831411170	-3.1818975699	34.2985367627	0.238467
C41	1.2140368575	-0.3478005172	32.2433300212	-0.292696
H42	-1.2466043426	-1.4523509067	33.4352140027	0.176273
N43	0.4488573285	-5.6781516584	35.6588858845	-0.276989
N44	3.0986426869	-6.3168742427	36.4000216410	-0.276989
N45	3.9187898095	-4.2543203730	34.8720651383	-0.276989
N46	1.2361344097	-3.3832150463	34.2856998002	-0.276989
Fe47	2.1751087947	-4.8471192658	35.3013198502	0.425000
H48	3.6397265093	-4.5970138349	38.3053640219	0.165195
H49	0.6146164615	-2.3374821273	36.3912458139	0.159761
H50	2.8430697625	-2.7919136913	39.9280966895	0.328887
H51	4.2467451855	-6.5573114858	33.4300010250	0.208630
H52	0.1355122674	-5.6284978918	32.9649365663	0.154493
H53	0.8633838827	-7.0028468966	30.9525158105	0.331262
H54	0.7600638035	-8.6704523913	37.3064450892	0.149311
H55	3.5870562216	-1.4559726303	32.8553294127	0.123769
H56	6.5144645763	-5.9699224250	36.5474862973	0.159482
H57	-2.2148318142	-3.5576469344	34.5826922848	0.198560
H58	7.7878610479	-0.7104617433	32.2147749139	0.147502
H59	8.2731175650	-2.2929914264	33.0225151671	0.159937
H60	5.4857200706	-1.0615661919	32.6816602697	0.140246
H61	7.2570469372	-8.9496295613	39.3957844083	0.151779
H62	5.4862963802	-8.7680336755	39.8914043546	0.161680
H63	6.8266151829	-7.9505769088	37.2503030047	0.123733
H64	4.0100335071	-10.0638624727	38.5495863659	0.098044
H65	2.7327612910	-9.1636237884	39.3650748215	0.098044
H66	2.3689667545	-10.0915887293	37.9047498233	0.098044
H67	8.1660145356	-2.9956206190	35.2032700204	0.100690
H68	7.9384912620	-4.6471427722	35.7621634222	0.100690
H69	8.1319283170	-4.3239521776	34.0382515860	0.100690
H70	-1.7381775272	-8.8385647174	37.4993707519	0.116140
H71	-2.8878358844	-8.6613100376	36.1705969314	0.116140
H72	-1.3216482278	-9.4381362872	35.8919764633	0.116140
H73	0.3003409076	0.2268552205	32.0703620493	0.101366
H74	1.9848331649	0.3546700968	32.5842350113	0.101366
H75	1.5444352287	-0.7431634898	31.2747706025	0.101366

c) heme a3 in state Fe(IV)=O (used for all calculations)

H38	-5.6190862081	-37.1675029949	20.5613171826	0.157824
N39	-6.8442915675	-36.1095730724	21.9895348831	-0.207126
H40	-7.6268375641	-36.7394697787	22.0790002153	0.316613
C41	-5.7335756117	-36.2909980218	21.1794846745	-0.138747
C42	-6.7010807601	-34.9238803102	22.6259582968	0.126215
H43	-7.4049957889	-34.5116211504	23.3320808668	0.105198
N44	-5.5503090507	-34.3588445223	22.2633277446	-0.335061
C45	-4.9452440472	-35.1883148349	21.3642905634	0.009826
H46	-3.9944289009	-34.9449101126	20.9190309940	0.086811
H47	0.2104785816	-34.9125771942	23.4328657543	0.160168

Fe48	-4.5184204499	-32.6416521663	22.8989580956	0.562061
N49	-2.8648006690	-33.7281769651	23.5761712994	-0.176399
N50	-5.3538540863	-32.7407635446	24.8568666373	-0.150605
N51	-5.9981861510	-31.3041549976	22.3726331576	-0.128398
N52	-3.5098871971	-32.2565793661	21.1056674638	-0.143865
C53	-1.7169820310	-33.9358358875	22.8478186742	0.206583
C54	-0.7880789194	-34.5975744229	23.7155774277	-0.290598
C55	-1.3746727485	-34.7646064123	24.9451906915	-0.063552
C56	-2.6944957702	-34.2251146125	24.8564708510	0.141559
C57	-4.8681833255	-33.5332831366	25.8795556988	0.067069
C58	-5.8658039558	-33.5129892003	26.9273318970	0.103347
C59	-6.9007876164	-32.7165331703	26.5310038895	-0.244257
C60	-6.5700017633	-32.2272437564	25.1999903716	0.123513
C61	-7.0682504703	-30.9200853988	23.1543873432	-0.017331
C62	-7.8484796438	-29.9605157689	22.4099332908	0.121088
C63	-7.2568045052	-29.7793826608	21.2031439710	-0.017751
C64	-6.0791782061	-30.6262827956	21.1599354178	0.044192
C65	-3.9463843793	-31.4023295552	20.1319859056	0.040356
C66	-2.9222565032	-31.3503961606	19.1140617013	0.167079
C67	-1.8971720724	-32.1559626221	19.5033762582	-0.385701
C68	-2.2764200639	-32.7262818211	20.7697046249	0.232631
C69	-1.5084520632	-33.5986734494	21.5199937226	-0.272957
H70	-0.5642673621	-33.9143238441	21.0845843150	0.140189
C71	-3.6557684319	-34.2007007233	25.8690995714	-0.212156
H72	-3.3668655714	-34.7215607305	26.7754817657	0.154845
C73	-7.3508851258	-31.3797296227	24.4232413008	-0.170412
H74	-8.2513585223	-31.0123450454	24.8996544773	0.158541
C75	-5.1727804438	-30.7339681631	20.1287022193	-0.173241
H76	-5.3804094412	-30.1468408745	19.2396427284	0.123366
C77	-0.7203503779	-35.3821699896	26.0968366526	0.401299
H78	0.2265947671	-35.9117239622	25.8461089048	-0.020246
O79	-1.1043938401	-35.3280839796	27.2558809620	-0.434365
C80	-5.7514258601	-34.2579373433	28.2295129919	-0.317330
H81	-4.7245613363	-34.5749254799	28.4271077037	0.104881
H82	-6.3742225561	-35.1614413140	28.2337424518	0.078587
H83	-6.0742325566	-33.6439578606	29.0778891479	0.111725
C84	-9.0845923040	-29.2873417000	22.9300720215	-0.278931
H85	-9.1282162297	-28.2434647254	22.6031701617	0.098724
H86	-9.1166819348	-29.2956137384	24.0230636382	0.077371
H87	-9.9993755422	-29.7844369728	22.5824207187	0.082733
C88	-7.6499638239	-28.9027824467	20.1007983288	-0.083917
H89	-6.8378605767	-28.5861745273	19.4469330884	0.121726
C90	-8.8730359307	-28.4174290989	19.8345425230	-0.378785
H91	-9.0308815006	-27.7465306164	18.9954392673	0.146703
H92	-9.7565007008	-28.7127535234	20.3888194855	0.158767
C93	-2.9973778799	-30.5271309401	17.8598757756	-0.300927
H94	-3.0532303148	-29.4538695223	18.0792403279	0.095647
H95	-3.8803334318	-30.7804087529	17.2598835186	0.078603
H96	-2.1151961718	-30.6908857339	17.2343759044	0.097210
C97	-8.0998014255	-32.3290711967	27.3565826724	0.319502
H98	-7.9081735958	-32.5670307831	28.4131962024	-0.016557
O99	-8.4723804561	-30.9546555075	27.2234886252	-0.613663
H100	-7.6842528621	-30.4249545479	27.4062866800	0.391412
H101	-8.9966290949	-32.8820456318	27.0496103428	0.034867
H102	-0.9698969828	-32.3452916349	18.9777700864	0.176710
O104	-3.7173545313	-31.3470686937	23.4935894406	-0.352657

d) CuB in PR state with Cu(II)-OH Tyr237-O- (used for Tyr237 deprotonated)

H1	-0.8264348977	-26.9346109600	26.1064242774	0.114395
N2	-2.2615098291	-28.5572664326	25.9604539222	-0.272336
C3	-1.8463080878	-27.2596556179	26.2418658027	-0.150851
C4	-3.5420971306	-28.6758556889	26.2606412562	0.239017
H5	-4.2047558321	-29.5248796211	26.1757183499	0.073658
N6	-4.0075359556	-27.4746763060	26.6779303239	0.018397
C7	-2.9594099957	-26.5594534038	26.6421526776	-0.139733
H8	-3.0851688944	-25.5565598424	27.0152909734	0.104747
H9	-7.4669767381	-24.5607221949	25.8510568872	0.096415
C10	-7.1688404465	-25.5584168702	26.1647318218	-0.355662
C11	-8.1455072571	-26.5516344439	26.3458615786	-0.025632
H12	-9.1974766433	-26.3140259532	26.2026918731	0.065093
C13	-7.7482593517	-27.8206578078	26.7208140444	-0.352689

H14 -8.4567905795 -28.6277836644 26.8823833952 0.101035
C15 -6.3642335389 -28.1607556020 26.8967965953 0.468574
O16 -5.9999644106 -29.3344808020 27.2440125686 -0.663003
C17 -5.8139427879 -25.8303667570 26.3081465185 -0.047149
H18 -5.0892850961 -25.0611900854 26.0485474013 0.091091
C19 -5.3883832049 -27.1014775020 26.7276621459 -0.156292
H20 0.7852770016 -33.3204222733 28.4027251972 0.151168
N21 -0.7357574042 -31.8060817970 28.5174237514 -0.138017
H22 -0.9905625006 -31.8829941978 29.4918222852 0.276540
C23 0.2228504796 -32.5635976410 27.8780136765 -0.274163
C24 -1.3050648697 -30.9706270807 27.6300361874 0.122950
H25 -2.0769605107 -30.2493471192 27.8480220333 0.070911
N26 -0.7548904222 -31.1951466430 26.4312355829 -0.347171
C27 0.2014882313 -32.1910163383 26.5710586696 0.066167
H28 0.7862817376 -32.5415473094 25.7365729296 0.093837
H29 3.7200734096 -31.2644666827 23.6833759693 0.099653
N30 1.9002902498 -31.5525148359 22.5596236380 -0.247127
H31 2.2419632600 -32.1739304486 21.8404850570 0.274181
C32 2.6731337940 -31.0195297081 23.5882368589 -0.084616
C33 0.6629101046 -31.0570231261 22.6444618907 0.048863
H34 -0.1651386774 -31.2846729282 21.9914555394 0.153108
N35 0.6137614190 -30.2121193382 23.6871710819 -0.100284
C36 1.8581144913 -30.1821177181 24.2830129771 -0.176187
H37 2.0642151958 -29.5799968425 25.1542071162 0.134585
Cu103 -1.0845506669 -29.8341269628 24.8938509096 0.345165
O106 -1.7536237560 -29.0975303164 23.2951111386 -1.015467
H107 -2.0035987608 -28.1759530489 23.4461681401 0.336828

e) CuB in PR state with Cu(II)-OH Tyr237-OH (used for Tyr237 protonated)

H1 -0.8903030011 -26.9122738263 26.1209729916 0.163635
N2 -2.2944940036 -28.5611061666 25.9699135968 -0.278232
C3 -1.9042401272 -27.2567284765 26.2489171452 -0.090344
C4 -3.5629998273 -28.7017951339 26.2571793843 0.114349
H5 -4.1555995250 -29.5928522102 26.1474667632 0.102382
N6 -4.0697007471 -27.5087776324 26.6699145053 0.115890
C7 -3.0311687802 -26.5748483892 26.6373482137 -0.178669
H8 -3.1643250919 -25.5708792226 27.0043431557 0.178374
H9 -7.4289088137 -24.4878232579 25.8404578774 0.143010
C10 -7.1677891328 -25.4920844851 26.1555426415 -0.156157
C11 -8.1532012886 -26.4580572992 26.3452393144 -0.046268
H12 -9.2010846602 -26.2104477308 26.2097203661 0.132988
C13 -7.7974924142 -27.7498716023 26.7265000525 -0.251665
H14 -8.5623231913 -28.5071009605 26.8828991695 0.148767
C15 -6.4586858996 -28.1008086512 26.8894203036 0.255685
O16 -6.0817660469 -29.3642035986 27.2643312960 -0.514649
C17 -5.8258681756 -25.8433330865 26.3050153643 -0.130014
H18 -5.0605022373 -25.1208508519 26.0473666949 0.142600
C19 -5.4570884075 -27.1219376779 26.7247512580 0.007477
H20 0.8816957634 -33.3136059777 28.3882102465 0.182608
N21 -0.6722779252 -31.8329388865 28.5058845328 -0.159156
H22 -0.9231420263 -31.9243597913 29.4806466206 0.319914
C23 0.3037379213 -32.5667058229 27.8661568171 -0.148849
C24 -1.2572878384 -31.0061882653 27.6199517629 0.065660
H25 -2.0434563230 -30.3068276519 27.8476507982 0.128051
N26 -0.6989880281 -31.2141920885 26.4215024389 -0.275759
C27 0.2781578347 -32.1892493218 26.5585397689 -0.003117
H28 0.8732574990 -32.5246893992 25.7253295240 0.111948
H29 3.7403608534 -31.2684578974 23.6634306582 0.154390
N30 1.9002898232 -31.5594265318 22.5739080537 -0.191458
H31 2.2255561008 -32.1901799057 21.8541887948 0.316181
C32 2.6929735597 -31.0204729792 23.5826282592 -0.074571
C33 0.6673097257 -31.0564169619 22.6763088870 0.081909
H34 -0.1710067264 -31.2888561002 22.0381824965 0.176658
N35 0.6401165951 -30.2000763563 23.7120840579 -0.220788
C36 1.8964372889 -30.1715901007 24.2847790246 -0.096136
H37 2.1260542437 -29.5638892556 25.1459890337 0.152475
Cu103 -1.0521976077 -29.8551141939 24.8881634956 0.593722
O106 -1.7456079573 -29.1113523064 23.3121095522 -0.760961
H107 -2.0223354844 -28.1942349722 23.4410356975 0.367948
H41 -6.8166525527 -29.9729106423 27.1083927072 0.420178

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